

Heme and a Five-Amino-Acid Hemophore Region Form the Bipartite Stimulus Triggering the *has* Signaling Cascade

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Bacterial cells sense the extracellular environment and adapt to that environment by activating gene regulation circuits, often by means of signaling molecules. The *Serratia marcescens* hemophore is a signaling molecule that acts as an extracellular heme-scavenging protein. The heme-loaded hemophore interacts with its cognate receptor (HasR), triggering transmembrane signaling and turning on transcription of hemophore-dependent heme uptake genes. We investigated the features of the holo-hemophore, the only HasR ligand known to act as an inducer. We used a hemophore mutant that does not deliver its heme and a HasR mutant that does not bind heme, and we showed that heme transfer from the hemophore to the receptor is necessary for induction. Using a hemophore mutant that does not bind heme and that blocks heme transport, we demonstrated that two molecules that do not interact (heme and the mutant hemophore) may nonetheless induce this system. These findings suggest that hemophore-mediated induction and heme transport involve different mechanisms. The hemophore region important for induction was precisely localized to amino acids 50 to 55, which lie in one of the two HasR-binding hemophore regions. This bipartite stimulus probably corresponds to a physiological process because heme is transferred to the receptor before apo-hemophore release. This bipartite regulation mechanism may allow the bacterium to adjust its heme transport mechanism to the perceived environmental heme concentration.

Most organisms, including bacteria, require iron. Bacteria encounter various types of environmental iron sources, including ferric and ferrous iron, iron-containing siderophores, heme, heme carrier proteins, and hemophores (10). In most cases, bacterial iron needs are covered by tightly regulated iron/heme uptake systems encoded by genes clustered in operons expressed only under iron deficiency conditions (2). In the presence of high intracellular iron concentrations, these operons are repressed by a common regulator, the iron-loaded Fur protein (3, 17).

However, not all iron acquisition systems are simultaneously induced in response to iron limitation. Many systems are also positively regulated by the availability of a specific iron source (9, 33). In gram-negative bacteria, iron/heme ligands are recognized by specific outer membrane proteins that play a key role in active transport and ligand-mediated positive regulation (13). These receptors have similar overall structures, with a membrane-inserted β barrel closed by an N-terminal plug region (15). The activity of these receptors is dependent on the TonB-ExbB-ExbD inner membrane protein complex, with which they interact via a short conserved region, named the TonB box, near the receptor's N terminus (6).

Ligand receptor binding, even in the absence of transport, is sufficient to induce transcription of the corresponding ligand uptake genes (12). This response to the extracellular environment is mediated by extracytoplasmic function (ECF) sigma factors and membrane-bound anti-sigma factors (4, 5, 21). Binding of the iron ligand to its cognate outer membrane

receptor releases the anti-sigma factor-mediated inhibition of the ECF sigma factor, inducing transcription of the target operons (18). The TonB-ExbB-ExbD complex is also required for transmembrane signaling. All the outer membrane receptors known to be regulated in this manner have a 50- to 100-amino-acid N-terminal regulatory extension beyond the TonB box; this extension is essential for sigma activation (32).

The *Serratia marcescens* hemophore-dependent heme-acquisition system (Has) is subject to this type of regulation (31). The Has system consists of a Fur-regulated operon encoding HasR, the hemophore-specific outer membrane receptor, HasA, the hemophore, and HasD and HasE, the specific inner membrane hemophore secretion proteins (16). The final gene of the *has* operon, *hasB*, encodes a TonB homolog involved in heme uptake via the Has system (29). Two genes immediately upstream of the *has* operon encode HasI, an ECF sigma factor, and HasS, an anti-sigma factor (31).

The heme-loaded hemophore is a globular 19-kDa protein with H32 and Y75 double iron coordination. The H83 residue is an alternative ligand of heme iron (24). The seven β strands form an antiparallel β -sheet on one face of the protein, and the four α helices are positioned on the other face (1).

The hemophore receptor, HasR, resembles other outer membrane receptors that are dependent on TonB for energy. It is predicted to have a similar three-dimensional structure, with a plug and a β barrel (26). HasR also functions as a heme receptor, taking up free and hemoglobin-bound heme, albeit less efficiently than it takes up hemophore-bound heme (16). HasR contains two histidine residues (H189 and H603) that have been shown to be conserved in several heme receptors (8) and to be required for free and hemophore-bound heme uptake (F. Huché, N. Izadi-Pruneyre, A. Lecroisey, R. Gilli, C. Wandersman, and P. Deleplaire, unpublished data). HasR

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
C600	F ⁻ <i>thr leu fhuA lacY thi supE</i>	Laboratory collection
XL10 Gold ultracompetent cells	Tet ^r $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte}$ [F' <i>proAB lacI^qZAM15 Tn10(Tet^r) Amy Cam^r</i>]	Laboratory collection
POP3	<i>araD139 $\Delta lacU169 rpsL relA thi$</i>	Laboratory collection
POP3BF1	POP3 <i>fur::Cm attλ::hasR-lacZ</i> (Amp ^r Cm ^r)	7
POP3 <i>hasR-lacZ hemA</i> (pAMhasISR)	POP3 <i>attλ::hasR-lacZ $\Delta hemA::Km$ pAMhasISR</i> (Amp ^r Km ^r Spc ^r)	This study
POP3 <i>hasR-lacZ hemA fur</i> (pAMhasISR)	POP3 <i>attλ::hasR-lacZ $\Delta hemA::Km fur::Cm$ pAMhasISR</i> (Amp ^r Km ^r Cm ^r Spc ^r)	This study
Plasmids		
pAMrbshasISR	pAM238 carrying <i>hasI</i> , <i>hasS</i> , and <i>hasR</i> , Spc ^r	7
pAMrbshasISR $\Delta 45-125$	pAM238 carrying <i>hasI</i> , <i>hasS</i> , and <i>hasR</i> $\Delta 45-125$, Spc ^r	7
pACYC-hasDE	pACYC184 <i>E. coli</i> cloning vector carrying <i>hasDE</i> , Cm ^r	24
X134pAM	pAM238 carrying <i>hasA</i> , Spc ^r	25
H32A pAM	pAM238 carrying <i>hasAH32A</i> , Spc ^r	24
Y75A pAM	pAM238 carrying <i>hasAY75A</i> , Spc ^r	24
76T pAM	pAM238 carrying <i>hasA76T</i> , Spc ^r	22
S51P pAM	pAM238 carrying <i>hasAS51P</i> , Spc ^r	22
S51A pAM	pAM238 carrying <i>hasAS51A</i> , Spc ^r	22
G95V pAM	pAM238 carrying <i>hasAG95V</i> , Spc ^r	22
T20 pAM	pAM238 carrying <i>hasAT20</i> , Spc ^r	22
T46 pAM	pAM238 carrying <i>hasAT46</i> , Spc ^r	22
T53 pAM	pAM238 carrying <i>hasAT53</i> , Spc ^r	22
T55 pAM	pAM238 carrying <i>hasAT55</i> , Spc ^r	22
T58 pAM	pAM238 carrying <i>hasAT58</i> , Spc ^r	22
T59 pAM	pAM238 carrying <i>hasAT59</i> , Spc ^r	22
T68 pAM	pAM238 carrying <i>hasAT68</i> , Spc ^r	22
T99 pAM	pAM238 carrying <i>hasAT99</i> , Spc ^r	22
T107 pAM	pAM238 carrying <i>hasAT107</i> , Spc ^r	22
T181 pAM	pAM238 carrying <i>hasAT181</i> , Spc ^r	22
H32AY75AH83A pAM	pAM238 carrying <i>hasAH32AY75AH83A</i> , Spc ^r	24
pBAD24- <i>hasR</i>	pBAD24 cloning vector carrying <i>hasR</i> , Amp ^r	22
pBAD24- <i>hasRH1H2</i>	pBAD24 cloning vector carrying <i>hasR</i> containing His 189-to-Ala and His603-to-Ala mutations, Amp ^r	Laboratory collection
pAMrbshasISRH1H2	pAM238 carrying <i>hasI</i> , <i>hasS</i> , and <i>hasR</i> containing His 189-to-Ala and His603-to-Ala mutations, Spc ^r	This study
H32AY75AH83AG95V pAM	pAM238 carrying <i>hasAH32AY75AH83AG95V</i> , Spc ^r	This study
H32AY75AH83AS51P pAM	pAM238 carrying <i>hasAH32AY75AH83AS51P</i> , Spc ^r	This study
T49 pAM	pAM238 carrying <i>hasAT49</i> , Spc ^r	This study
T50 pAM	pAM238 carrying <i>hasAT50</i> , Spc ^r	This study
T56 pAM	pAM238 carrying <i>hasAT56</i> , Spc ^r	This study
G95P pAM	pAM238 carrying <i>hasAG95P</i> , Spc ^r	This study

binds free heme with a K_d around 1 μ M. It binds apo- and holo-hemophores at the same or overlapping sites with similar apparent K_d values (5 nM) (24). This interaction is mediated by two independent hemophore-binding regions, β strands 51-60 and 95-105 (22). The binding of heme or hemophore and the transfer of heme from the hemophore to the receptor are energy independent. In contrast, heme transport and the release of heme-free hemophore require energy, with hemophore release demanding the most energy (23).

The *has* operon is induced by a heme-loaded hemophore (holo-HasA). This signaling process is dependent both on HasR and on HasI and HasS (31) and has been fully reconstituted in *Escherichia coli* (7). Neither heme nor apo-HasA can induce *hasR* expression, raising questions about the nature of the induction stimulus (7).

We used a collection of HasR and HasA mutants and showed that induction may result from the concomitant addition of two molecules that do not interact directly, heme and a mutant hemophore that does not bind heme. The apo-hemo-

phore region required for induction is a five-amino-acid region located between positions 50 and 55 in one of the two HasR-binding regions of the hemophore.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Bovine hemoglobin, δ -aminolevulinic acid, and 2,2'-dipyridyl were obtained from Sigma. The hemoglobin concentration was calculated based on the levels of heme monomer. Hemoglobin and δ -aminolevulinic acid solutions were filter sterilized by using filters with 0.45- μ m pores. Bacteria were grown aerobically at 37°C in LB rich medium or in M63B1 or M9 minimal medium (28) supplemented with 0.4% glucose or 0.4% glycerol as a source of carbon. When required, ammonium iron(III) citrate was added to a final concentration of 5 μ M. The iron chelator 2,2'-dipyridyl was used at a final concentration of 0.2 mM, and δ -aminolevulinic acid was used at a final concentration of 20 μ g \cdot ml⁻¹. Antibiotics were added to the following final concentrations: ampicillin, 50 μ g \cdot ml⁻¹; spectinomycin, 100 μ g \cdot ml⁻¹; chloramphenicol, 20 μ g \cdot ml⁻¹; and kanamycin, 25 μ g \cdot ml⁻¹. Solid media contained 1.5% Difco agar.

Genetic techniques. Cells were transformed by the calcium chloride method (27) or by electroporation (14).

TABLE 2. Primers used for site-directed mutagenesis

Mutation	Forward primer	Reverse primer
H32AY75AH83AG95V	5'-ATTCCTGTCTTCCTCGTCGACGGTTGAGCGGT-3'	5'-ACCGCTCAAACCGTCGACGAAGGACAGGGAATC-3'
H32AY75AH83AS5P	5'-TTCTATGGCGGCAGCCTGCCCGGTAGTCAGTA TGCC-3'	5'-ATGGCATACTGACTACCGGGCAGGCTGCCGCC ATAG-3'
T50	5'-TTCTATGGCGGCAGCCTGGGGTACCCAGTATGT CCGGTAGTCAGTATGCC-3'	5'-ATACTGACTACCGGACATACTGGGGTACCCAGGC TGCCGCCATAGAAGCC-3'
T56	5'-TCCGGTAGTCAGTATCCCGGTACCCCACTATGA TCAGTAGCACGGCAAACC-3'	5'-TTTGCCGTGCTACTGATCATACTGGGGTACCCGGC ATACTGACTACCGG-3'
T49	5'-TTCTATGGCGGCAGCGGGTACCCAGTATGCTGT CCGGTAGTCAGTATGCC-3'	5'-ATACTGACTACCGGACAGCATACTGGGGTACCCGC TGCCGCCATAGAAGCC-3'
G95P	5'-ATTCCTGTCTTCCTCCCGACGGTTTGTAGCGGTG-3'	5'-ACCGCTCAAACCGTCGGGGAAGGACAGGGAATC-3'

DNA manipulation and molecular cloning. Large-scale plasmid DNA preparations were obtained with a QIAfilter plasmid midi kit (QIAGEN Inc.), used according to the manufacturer's instructions. Small-scale plasmid DNA preparations were obtained with a QIAprep spin miniprep kit (QIAGEN Inc.), used according to the manufacturer's instructions. Restriction, modification, and ligation were carried out according to the recommendations of the kit manufacturers. Nucleotide sequencing was performed by Genome Express.

Site-directed mutagenesis. Site-directed mutagenesis was carried out with a Stratagene QuickChange XL site-directed mutagenesis kit (catalog no. 200516) and was verified by sequencing. Mutants T49, T50, T56, G95P, H32AY75AH83AS51P, and H32AY75AH83AG95V were generated in pAM238-*hasA* by using the primers described in Table 2. The pentapeptide insertions generated in all the HasA mutants used are described in Table 3.

Plasmid construction. We constructed pAMrshasISRHIH2 by replacing the mutant copy of the *hasR* gene (D1, a large part of the *hasR* sequence deleted) in pAMrshasISRDI with the *hasR* gene of interest (H1H2). The donor plasmid pBAD24hasRHIH2 was cut with FseI and KpnI, which cleaved the ends of the *hasR* gene. The FseI-KpnI fragment containing *hasRHIH2* was then purified and ligated with the recipient plasmid, pAMrshasISRDI, which was previously digested with FseI and KpnI. The resulting construct was used to transform cells, and clones containing the pAM recombinant plasmid (pAMhasISRHIH2) were selected on plates containing spectinomycin and screened in digestion experiments.

Electrophoresis and immunological techniques. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie blue staining. Dot blot HasA binding assays were performed with cultures of C600(pBAD24) and C600(pFR2hasR), as previously described (24). Briefly, aliquots (50 μ l; approximately 10^7 CFU) of washed cell pellets were applied to nitrocellulose filters. Dot blotting with serial dilutions of wild-type or mutant apo-HasA proteins or no HasA was performed as described by Letoffe et al. (25).

Preparation of apo-HasA proteins and loading with heme. Apo-HasA proteins were obtained from the culture supernatant of strain POP3(pACYC184-*hasDE*, pAM 238-*hasA*) grown at 30°C in M9 glycerol medium supplemented with 5 μ M ammonium iron(III) citrate. The supernatant was collected and concentrated by

precipitation with 65% ammonium sulfate. A 5- to 10-ml aliquot of the sample was then extensively dialyzed in a cassette (Slide-A-Lyser dialysis cassette [extra strength] from Pierce) against 1 liter of TN buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing protease inhibitors (Complete EDTA-free protease inhibitor cocktail tablets from Roche). Aliquots of the dialyzed preparation were frozen and stored at -20°C . The heme content of the protein, as determined from absorbance at the Soret band wavelength, was less than 0.5% in all cases. The purity of protein preparations was more than 99%, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Apo-HasA was loaded with heme by mixing 1 ml of 5×10^{-6} M apo-HasA in TN buffer with 1 ml of 4.5×10^{-6} M heme in TN buffer and incubating the preparation for 15 min at room temperature. Holo-HasA heme loading was determined based on Soret band intensity.

β -Galactosidase assay. β -Galactosidase was assayed as described by Pardee et al. (30).

RESULTS

Heme transfer from HasA to HasR is required for induction of the *has* signaling cascade. (i) Study of a HasA mutant affected in heme release. One mutant from our pentapeptide insertion mutant collection, HasA 76T, had the following properties: pentapeptide insertion between residues 76 and 77 (Fig. 1), heme binding with a K_d of 10^{-9} M (the K_d for wild-type HasA was 10^{-11} M), and binding to the receptor with an affinity similar to that of the wild-type protein (22). These properties and the results of a circular dichroism analysis suggested that the mutant and wild-type proteins had similar conformations (data not shown). However, the mutant was unable to supply heme to cells expressing

TABLE 3. Pentapeptides inserted in the HasA mutant forms^a

Name of insertion	Pentapeptide inserted
T20 (in vivo)	L ₂₀ -GYPYL-G ₂₁
T46 (in vivo)	Y ₄₆ -GGYPY-G ₄₇
T49 (in vitro)	S ₄₉ -GYPSM-L ₅₀
T50 (in vitro)	L ₅₀ -GYPSM-S ₅₁
T53 (in vivo)	S ₅₃ -RGTPS-Q ₅₄
T55 (in vivo)	Y ₅₅ -GVPQY-A ₅₆
T56 (in vitro)	A ₅₆ -GYPSM-I ₅₇
T58 (in vivo)	S ₅₈ -RGTPS-S ₅₉
T59 (in vivo)	S ₅₉ -RGTPS-T ₆₀
T68 (in vitro)	V ₆₈ -GYPSM-A ₆₉
T99 (in vitro)	S ₉₉ -GYPSM-G ₁₀₀
T107 (in vitro)	S ₁₀₇ -GYPSM-I ₁₀₈
T181 (in vivo)	D ₁₈₁ -RGTPD-S ₁₈₂

^a The numbers indicate the position of the last amino acid preceding the pentapeptide insertion. Mutants obtained with the Tn430 transposon are indicated by "in vivo" (22); "in vitro" indicates the mutants obtained by site-directed mutagenesis leading to a pentapeptide insertion.

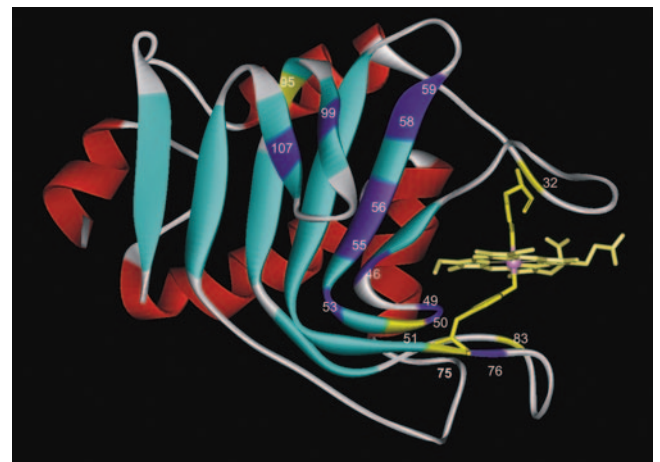


FIG. 1. Location of single amino acid change (yellow) and insertion site of the pentapeptide (purple) in the three-dimensional structure of HasA.

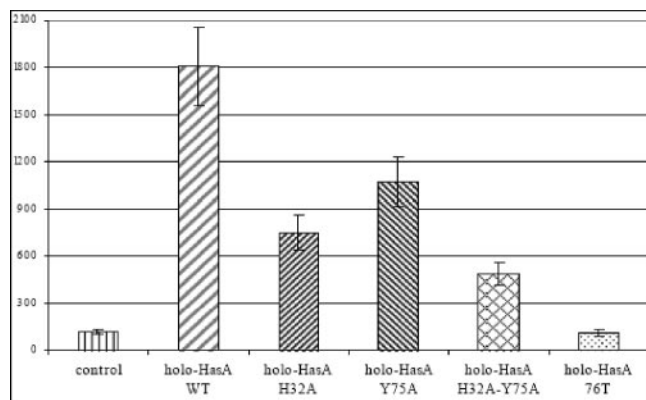


FIG. 2. Effect of HasA mutants, affected in heme binding or release, on *hasR-lacZ* expression. Strain POP3BF1(pAMrbshasISR) was grown in M63B1 glucose medium in the presence and absence of the heme-loaded form of wild-type or mutant hemophores at a concentration of 10^{-7} M. β -Galactosidase activity was assayed as described in Materials and Methods. The β -galactosidase activities are means of at least three experiments; the error bars indicate one standard deviation. WT, wild type.

HasR. Also, it displayed no heme transfer from holo-HasA 76T to apo-HasR “in vitro” (Huché, unpublished data). The mutation therefore seemed to involve heme release. All our studies of *hasR-lacZ* induction were carried out in *E. coli* strain POP3BF1 (POP3 *fur::Cm att λ ::hasR-lacZ*) and its derivatives. Addition of holo-HasA 76T to strain POP3BF1(pAMrbshasISR) in M63 glucose medium did not induce expression of the *hasR-lacZ* fusion, whereas a holo-HasA wild-type hemophore induced expression of the fusion by a factor of about 15, as previously reported (7)

(Fig. 2). The heme-loading status of holo-HasA 76T was monitored during the incubation; unlike wild-type holo-HasA, the mutant protein continued to display 100% heme loading throughout the experiment (data not shown). HasA 76T had a lower affinity for heme than the wild-type protein, and the insertion affected the heme pocket. We therefore evaluated the induction activities of heme pocket mutants with affinities for heme of 10^{-11} M (wild-type HasA and HasAH32A), 5×10^{-9} M (HasAY75A), and 10^{-7} M (HasAH32AY75A) (24). These mutants supply heme to HasR-expressing cells (24). Mutant proteins loaded with heme were added to strain POP3BF1(pAMrbshasISR). All the heme pocket mutants tested induced the *hasR-lacZ* fusion about 30% less efficiently than the wild type, implying that the heme pocket per se is not involved in *hasR* induction (Fig. 2).

(ii) **HasR mutant study.** We used a HasR mutant unable to use free or hemophore-bound heme. This mutant carries the mutations H189A and H603A (H1H2) and has no affinity for heme (Huché et al., unpublished). *E. coli* strain POP3BF1 was transformed with pAMrbshasISR (a plasmid containing wild-type *hasR*) or pAMrbshasISRH1H2 (encoding the HasR mutant protein). Production of the mutant protein was assessed by immunodetection with anti-HasR antibodies (data not shown). Similar amounts of the mutant and wild-type proteins were produced. The presence of the mutant at the cell surface and its reactivity with HasA were confirmed by dot blot assays with whole cells (data not shown). Strains POP3BF1(pAMrbshasISR) and POP3BF1(pAMrbshasISRH1H2) were grown in M63 glucose medium. They displayed β -galactosidase activity at subinduction levels. Holo-HasA increased *hasR-lacZ* expression by a factor of about 15 with the wild-type HasR receptor (Fig. 3), as previously reported (7). In contrast, the *hasR-lacZ*

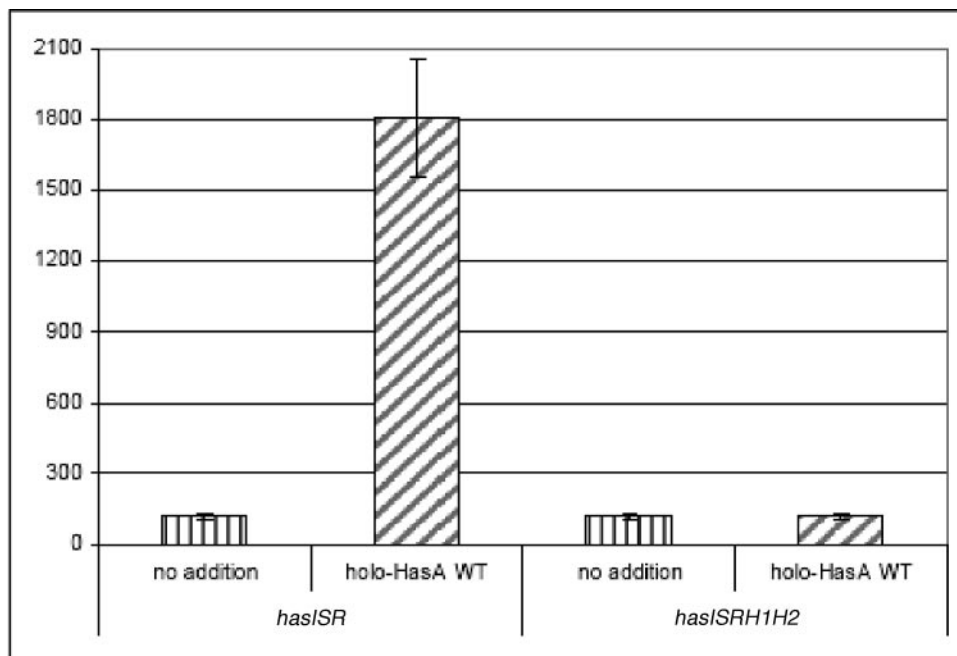


FIG. 3. Effect of heme transfer from the hemophore to the receptor on *hasR-lacZ* expression. Strain POP3BF1(pAMrbshasISR) and a derivative of this strain containing a mutant copy of *hasR* (H1-H2) were grown in M63B1 glucose medium in the presence and absence of wild-type heme-loaded HasA (holo-HasA) at a concentration of 10^{-7} M. β -Galactosidase activity was assayed as described in Materials and Methods. The β -galactosidase activities are means of at least three experiments; the error bars indicate one standard deviation. WT, wild type.

fusion was not induced following the addition of holo-HasA to cells producing HasRH1H2. Heme release from the hemophore was measured by determining the decrease in the cell culture supernatant holo-HasA Soret band intensity during the course of the induction experiment. Holo-hemophore incubated with the strain expressing the wild-type receptor was entirely unloaded, but holo-hemophore incubated with the strain expressing the mutant receptor displayed 100% loading throughout the experiment (data not shown).

This set of experiments with a HasA mutant unable to deliver heme and a HasR mutant unable to accept heme showed that holo-hemophore binding to HasR is not sufficient for induction; heme must reach the receptor for initiation of the *has* signaling cascade. However, heme alone is not sufficient for induction, suggesting that all or part of the HasA protein is also required for induction. We therefore tried to identify the hemophore regions required for *hasR* induction.

Identification of hemophore regions required for *hasR* induction. The interaction between HasA and HasR involves two HasA regions, a region between positions 51 and 60 and a region between positions 95 and 105. Single mutants S51P and G95V both bound HasR with high affinity, whereas the double mutant had no measurable affinity. All these mutants bound heme with high affinity and had the same overall structure as the wild type (22).

We assessed the ability of the two previously described single mutants, S51P and G95V (22), to induce *hasR* expression. Holo-HasA S51P and holo-HasA G95V were added to the test strain POP3BF1(pAMrbshasISR). Addition of holo-HasA G95V induced expression of the *hasR-lacZ* fusion about sevenfold. Holo-HasA S51P induced *hasR-lacZ* expression by a factor of two (Fig. 4A). Other proteins with single changes in the two HasA binding regions were also tested, and holo-HasA S51A (not affected in HasA binding to HasR) fully induced the *hasR-lacZ* fusion (data not shown). Holo-HasA G95P induced the *hasR-lacZ* fusion to an extent similar to the extent observed with holo-HasA G95V (Fig. 4A). Thus, the binding region lying between residues 95 and 105 can be deformed by a proline without significantly changing the HasA induction properties.

Two other mutants from the laboratory collection with pentapeptide insertions near residues 95 and 105 (Table 3), HasAT99 and HasAT107, (22), were tested for *hasR* induction. Both of these mutants induced the *hasR-lacZ* fusion to an extent similar to the extent observed with holo-HasA G95V (Fig. 4A). The binding region of HasA between residues 51 and 60 is therefore clearly more important for induction than the binding region between residues 95 and 105 is.

Other HasA mutants carrying pentapeptide insertions at various positions in the HasA protein outside the two binding regions (positions 51 to 60 and 95 to 107) were also loaded with heme and tested. All of these mutants induced *hasR* to an extent similar to the extent observed with wild-type holo-HasA (Fig. 4B), indicating the absence of significant involvement of any other HasA region in the initiation of signal transduction.

Scanning the 51-60 binding region for induction properties with pentapeptide insertion mutants. The failure of the HasA S51P mutant to induce *hasR-lacZ* suggested that the region between residues 51 and 60 in HasA is important for the initiation of *has* signaling.

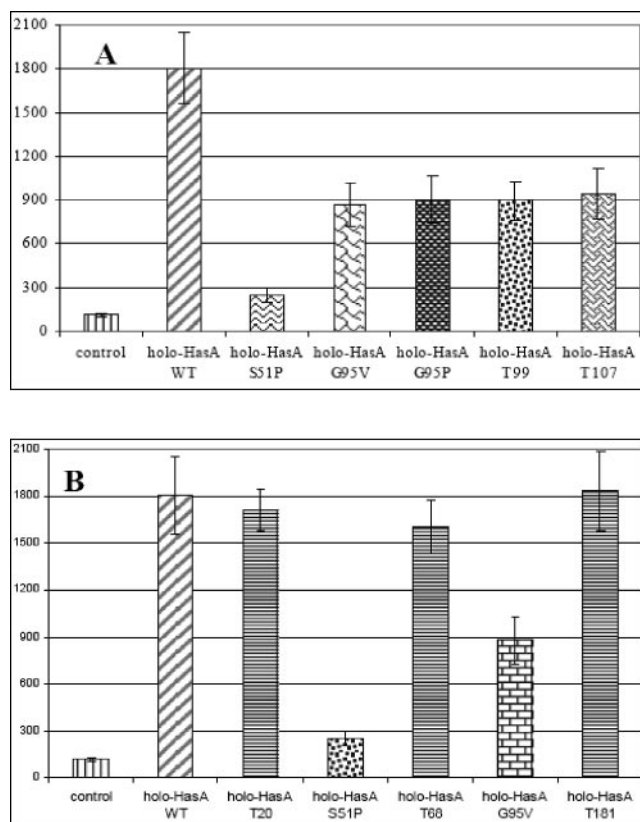


FIG. 4. Role of the HasR-binding regions of HasA in *hasR-lacZ* induction. Strain POP3BF1(pAMrbshasISR) was grown in M63B1 glucose medium in the presence and absence of heme-loaded wild-type or variant hemophores having mutations in the binding regions of HasA (A) or all along HasA (B) at a concentration of 10^{-7} M. β -Galactosidase activity was assayed as described in Materials and Methods. The β -galactosidase activities are means of at least three experiments; the error bars indicate one standard deviation. WT, wild type.

We studied a collection of mutants (22) obtained by insertion of a pentapeptide into this region (see Materials and Methods) for precise identification of the residues important for holo-HasA-dependent *hasR-lacZ* induction. These mutant proteins were loaded with heme and tested for *hasR-lacZ* induction in the test strain. Little or no induction of the *hasR-lacZ* fusion was observed with holo-HasA T50, holo-HasA T53, and holo-HasA T55. In contrast, holo-HasA T46, holo-HasA T49, holo-HasA T56, holo-HasA T58, and holo-HasA T59 all strongly induced expression of the *hasR-lacZ* fusion (Fig. 5). Holo-HasA T46 and holo-HasA T49 induced *hasR-lacZ* expression to an extent similar to the extent observed with the wild type, indicating that the region upstream of residue 50 is not required for *hasR* induction. Pentapeptide scanning analysis therefore implicated residues 50 to 55 of the 51-60 β -strand in the initiation of *has* signaling.

Noninteracting heme and HasA are able to initiate the *has* signaling cascade. Both heme delivery and residues 50 to 55 of the 51-60 binding region of HasA were required for *hasR-lacZ* induction. We investigated whether these two induction factors could act separately by determining whether supplying free heme with HasA led to *hasR* induction. To prevent heme

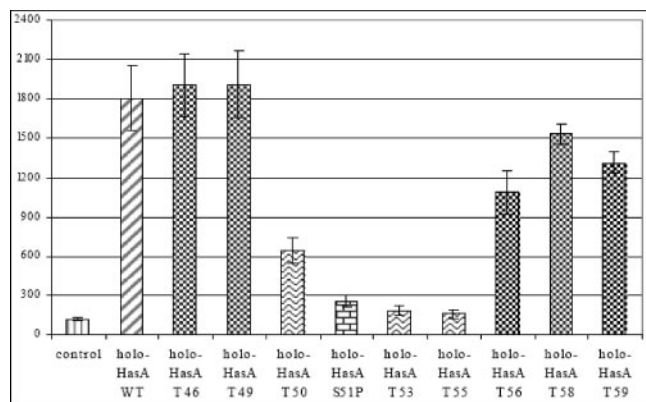


FIG. 5. Effect of pentapeptide insertions in HasA binding region 51-60 on *hasR-lacZ* expression. Strain POP3BF1(pAMrbshasISR) was grown in M63B1 glucose medium in the presence and absence of heme-loaded wild-type or mutant HasA hemophore at a concentration of 10^{-7} M. β -Galactosidase activity was assayed as described in Materials and Methods. The β -galactosidase activities are means of at least three experiments; the error bars indicate one standard deviation. WT, wild type.

binding to HasA, a HasA H32AY75AH83A triple mutant unable to bind heme was used.

Addition of HasA H32AY75AH83A or heme alone had no effect on *hasR-lacZ* expression in our test strain (Fig. 6). Full *hasR-lacZ* expression, as observed with wild-type holo-HasA, was induced in the presence of both heme and HasA H32AY75AH83A. The *has* signaling cascade may therefore be initiated in two ways: by holo-HasA and heme transfer to HasR or by concomitant addition of heme and the mutant hemophore unable to interact with heme. Heme binds to HasR on cells loaded with HasA H32AY75AH83A, inducing *hasR-lacZ*.

HasA H32AY75AH83A has been used to study heme uptake via HasR (23). HasA H32AY75AH83A does not bind heme and blocks free heme uptake (23). We compared the abilities of the wild-type and H32AY75AH83A hemophores to block heme uptake in the POP3 *hasR-lacZ hemA*(pAMrbshasISR) strain and its *fur* derivative. The H32AY75AH83A hemophore blocked heme uptake in both POP3 *hasR-lacZ hemA*(pAMrbshasISR) and its *fur* derivative. The H32AY75AH83A hemophore can therefore induce *hasR* expression while blocking heme uptake; heme transport is not required for *hasR* induction. Like HasA H32AY75AH83A, HasA 76T blocks heme transport. However, unlike HasA H32AY75AH83A, HasA 76T does not induce the *hasR-lacZ* fusion in the presence of heme.

HasA regions involved in induction in the presence of separately provided heme and hemophore. We introduced S51P and G95V mutations separately into HasA H32AY75AH83A to investigate the involvement of HasA binding regions 51-60 and 95-105 in the induction of *has* signaling in the presence of noninteracting heme and hemophore. The HasA quadruple mutants H32AY75AH83AS51P and H32AY75AH83AG95V were tested for *hasR-lacZ* induction (Fig. 6). Neither 10^{-5} M heme nor 10^{-7} M apo quadruple mutant supplied separately induced *hasR*. Concomitant addition of HasA H32AY75AH83AG95V and heme induced *hasR* by a factor of five, whereas

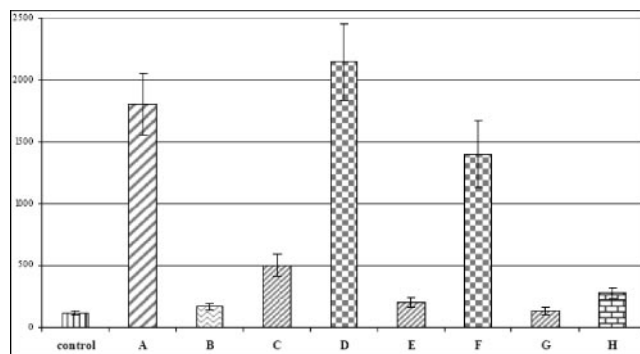


FIG. 6. Induction of *hasR-lacZ* by two noninteracting molecules, either heme and triple mutant HasA H32AY75AH83A, heme and quadruple mutant HasA H32AY75AH83AS51P, or heme and quadruple mutant HasA H32AY75AH83AG95V. Strain POP3BF1(pAMrbshasISR) was grown in M63B1 glucose medium in the presence and absence of heme-loaded HasA (holo-HasA) at a concentration of 10^{-7} M. β -Galactosidase activity was assayed as described in Materials and Methods. The β -galactosidase activities are means of at least three experiments; the error bars indicate one standard deviation. Bar A, wild-type holo-HasA at a concentration 10^{-7} M; bar B, heme at a concentration of 10^{-5} M; bar C, apo-HasA H32AY75AH83A at a concentration of 10^{-7} M; bar D, apo-HasA H32AY75AH83A at a concentration of 10^{-7} M plus heme at a concentration of 10^{-7} M; bar E, apo-HasA H32AY75AH83AG95V at a concentration of 10^{-7} M; bar F, apo-HasA H32AY75AH83AG95V at a concentration of 10^{-7} M plus heme at a concentration of 10^{-7} M; bar G, apo-HasA H32AY75AH83AS51P at a concentration of 10^{-7} M; bar H, apo-HasA H32AY75AH83AS51P at a concentration of 10^{-7} M plus heme at a concentration of 10^{-7} M.

concomitant addition of HasA H32AY75AH83AS51P and heme did not induce *hasR* expression. In both cases, the amounts of the mutant proteins were similar to the amounts of the wild-type hemophore in controls at the end of the incubation period, as shown by immunodetection (data not shown). The quadruple mutant HasA H32AY75AH83AS51P interacted normally with HasR, as shown by dot blotting (data not shown). Thus, residue 51 is also required for *hasR* induction in the absence of an interaction between heme and the hemophore. The same region (region 50-55) of HasA is probably required for induction by holo-HasA and for induction by noninteracting, separately supplied heme and hemophore.

DISCUSSION

Expression of the *has* operon of *S. marcescens*, which allows heme acquisition, is positively regulated by a transmembrane signaling cascade. This cascade is initiated by the binding of heme-loaded hemophore to HasR, its cognate receptor, which leads to specific sigma factor activation. HasR has a number of ligands, heme, apo-hemophore, and holo-hemophore, but only holo-hemophore initiates the signaling cascade. This raises questions concerning the nature of the inducing stimulus. This stimulus may be a particular holo-HasA conformation. Indeed, the three-dimensional structures of holo-HasA and apo-HasA indicate that heme binding to HasA substantially modifies the conformation of the heme pocket, without changing the α - β fold of the protein (1) (PDB accession number PDB ID 1YBJ). Alternatively, induction may result from the extraction of heme from HasA, heme landing on HasR, or empty hemophore recycling.

We used two types of mutants blocked in heme transfer from HasA to HasR: HasA mutants unable to deliver heme and a HasR mutant unable to receive heme. Both types of mutation abolish holo-HasA-mediated *hasR* induction. Thus, no particular holo-HasA conformation acts as the stimulus for induction. Interaction between heme and the receptor is necessary for induction. However, no induction was observed with heme alone, suggesting that induction requires either heme transfer or the concomitant presence of heme and apo-HasA on the receptor. The addition of heme together with a HasA mutant unable to bind heme (HasA H32AY75AH83A) led to full *hasR* induction, demonstrating that a direct interaction between heme and the hemophore is not required to trigger induction. Thus, the extraction of heme from the hemophore is not the stimulus required for induction.

Our findings also indicate that HasA H32AY75AH83A binding to HasR does not prevent heme from reaching HasR. However, HasA H32AY75AH83A binding to the receptor blocks free heme uptake (23). Similar results were obtained with a *hemA* isogenic mutant of POP3BF1(pAM*hasISR*), the strain used for the induction experiments (data not shown). This induction by heme in the presence of HasA H32AY75AH83A led us to reject our initial hypothesis that HasA H32AY75AH83A prevents heme from gaining access to HasR (23). Purified heme-free HasA H32AY75AH83A-HasR complexes bind heme in vitro, confirming that heme can reach a hemophore-loaded receptor (N. Izadi, personal communication). HasA H32AY75AH83A may remain on the receptor, blocking heme transport but not heme binding. The mechanism by which heme transport is inhibited remains unclear. It is possible that the wild-type hemophore dissociates from the receptor after its heme is transferred, whereas no such dissociation is observed with a mixture of heme and the H32AY75AH83A mutant hemophore. Hemophore recycling may require heme transfer from the hemophore to the receptor. This process is not possible for HasA molecules not loaded with heme. The induction-incompetent HasA 76T molecule, which cannot deliver its heme, also inhibits heme uptake. These findings are consistent with the hypothesis that heme transfer is necessary for hemophore recycling. Alternatively, a small amount of heme may reach HasR in the presence of HasA H32AY75AH83A, and this may be sufficient for induction but not growth.

Whatever the mechanism of inhibition, this study demonstrated that induction and transport are distinct mechanisms, as they are in several other systems (12, 20).

The hemophore (plus heme) is required for induction, implicating HasR-binding regions of HasA (S51 to Y60 and G95 to P105) in induction. Mutations affecting the region between residues 95 and 105 of HasA halved induction. Mutations in the 50-55 sequence of HasA abolished induction. Mutation between Y55 and T60 did not decrease induction but modified the binding properties of HasA (22). Thus, only one half of the 51-60 binding region, corresponding to a bend, is required for induction. We investigated the roles of the two HasA binding regions in the presence of independently supplied heme and apo-HasA, using the quadruple mutants HasA H32AY75AH83AS51P and HasA H32AY75AH83AG95V. These mutants have mutations in one binding region and are unable to bind heme. HasA H32AY75AH83AS51P did not induce *hasR* in the presence of heme, demonstrating that the 51-60 binding region of HasA is important for induction both by holo-

HasA and by heme plus apo-HasA. A synthetic peptide corresponding to the β strand between residues 51 and 60 competed with the corresponding HasA region for binding to the receptor (22). Nevertheless, concomitant addition of heme and this HasA peptide did not induce *hasR* (data not shown). This suggests that the 51-60 region adopts a particular conformation for induction that is different from the conformation required for binding and that the synthetic peptide does not adopt this conformation. The slightly-weaker-than-wild-type induction by HasA proteins carrying mutations in the 95-105 binding site is consistent with the hypothesis that HasA protein integrity is required to ensure the correct conformation of residues 50 to 55.

We broke the induction stimulus down into two elements, which may correspond to physiological steps in heme delivery. Studies with purified HasA-HasR complexes have shown that heme transfer in vitro requires neither an energy supply nor complex dissociation (Huché et al., unpublished). HasA/HasR complex dissociation does not occur in vitro, but it is the most energy-demanding step in vivo. Thus, heme is transferred from HasA to HasR before release of the apo-hemophore from the receptor, and this is the signal perceived by the bacterium. TonB is required for *hasR* induction, acting at and playing a role in a subsequent step. According to the FecA model, ligand binding generates a TonB-dependent conformational change in the receptor regulatory region, releasing the sigma factor from anti-sigma factor inhibition (12).

The bipartite regulation of the *has* system allows the bacterium to perceive the environmental heme concentration and to adjust its heme transport mechanisms accordingly. Under iron limitation conditions and in the absence of heme, *S. marcescens* produces basal levels of HasR and HasA, which form complexes at the cell surface. This may prevent hemophore dilution and inactivation in the surrounding medium. In the presence of low concentrations of heme, preformed HasA-HasR complexes switch the system on, facilitating heme scavenging at nanomolar concentrations. When the relative heme/HasA ratio is high, such as during growth in the bloodstream, heme transport leads to apo-hemophore recycling, with free heme binding more frequently than loaded hemophores to receptors. This binding of free heme does not induce the signaling cascade. Heme is taken up via residual HasR receptors or by other *S. marcescens* heme uptake systems induced in iron-chelated medium.

Like HasA, several iron ligands (ferric dicitrate, pyochelin, and pyoverdine) (19, 34) bind to their specific outer membrane receptors as iron-free molecules. Other ligands are positively regulated by transmembrane signaling mechanisms, like that of the canonical Fec system (11). For citrate, as for HasA, only the iron-loaded form triggers the signal (11, 31). Crystal structures of two siderophore receptors have shown that very different conformational changes are induced by the binding of apo- and holo-siderophores (34). However, the iron carried by the siderophore is not bound directly to the receptor, and the loaded molecule is transported as a whole. Thus, for siderophore-dependent iron transport, the metal ion and its organic chelator do not have different functions in initiation of the signaling cascade.

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